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(54) Title: METHOD OF PROMOTING KERATINOCYTE PROLIFERATION

(57) Abstract

The present invention provides methods, improved cell culture medium and kits for promoting epithelial stem cell and keratinocyte proliferation and differentiation by growth in the presence of angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

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METHOD OF PROMOTING KERATINOCYTE PROLIFERATION

Cross Reference

This application is a continuation in part of U.S. Application Serial No. 60/074,105 filed February 9, 1998.

Field of the Invention

This present invention relates to methods to accelerate the proliferation of epithelial stem cells and keratinocytes, to promote more rapid and efficient cellular response to stratified epithelial injury, and to decrease scarring and loss of function at the injury site.

Background of the Invention

The epidermis is composed of a continually renewing stratified layer of epithelial cells, called keratinocytes. (U.S. Patent No. 5,693,332, hereby incorporated by reference in its entirety). The basal layer of the epidermis, which is in direct contact with the basal lamina and dermal connective tissue, contains epithelial stem cells that divide and give rise to keratinocytes, which produce keratin as they differentiate and are "pushed" to the surface of the epidermis. (*Id.*) As the keratinocytes approach the surface of the epidermis, the cells die and the keratin contributes to the cornified skin surface, which is substantially impermeable to water and acts to prevent bacterial infection. (*Id.*) This process is highly conserved and can be faithfully monitored via a panel of biochemical and morphological markers. (Coulombe, *Biochem. and Biophys. Res. Commun.* 236:231-238 (1997); hereby incorporated by reference in its entirety).

This superficial epidermal layer consists of stacks of terminally differentiated, highly flattened squamous cells ("squames"). (Coulombe, 1997). Due to the constant frictional forces sustained at the surface of the body, squames are continuously being shed from the skin surface. Under normal conditions, the epidermis is a self-renewing tissue in which the rate of cell loss at its surface is balanced by the rate of cell production in its progenitor compartment. (*Id.*; Watt, *Curr. Opinion Cell Biol.* 1:1107-1115 (1989); Fuchs, *J. Cell Sci. Suppl.* 17:197-208 (1993); all references hereby incorporated in their entirety).

The dermis is a well vascularized tissue that provides support for the epidermis. The dermis contains fibroblasts, which produce various elements of the connective tissue, including extracellular matrix proteins such as collagens, fibronectin and elastin that contribute to the strength and flexibility of the skin. (U.S. Patent No. 5,693,332). Blood vessels present in the dermis transport nutrients to the epithelial cells in the epidermis and carry away waste products of cell metabolism. The basement membrane serves, in part, to attach the epidermis to the dermis. The skin also contains various accessory organs such as hair follicles and sweat glands.

An injury to the skin due, for example, to a laceration, a puncture or a burn results in a wound that can extend into or through the skin. If the wound is fairly small and localized, normal healing processes can close the wound and restore normal function to the tissue. (*Id.*) In some cases, however, an injury results in a deep wound or a wound that affects a large area. Such wounds can require clinical intervention for healing to occur. For example, a burn that covers a significant portion of the body requires, at a minimum, extensive cleaning of the injured tissue and application of a dressing to prevent infection of the tissue, and may be accompanied by scar formation,

possibly producing disfigurement and loss of function at the injured region. (*Id.*) In addition, skin grafting using undamaged skin from the patient or a skin substitute often is required.

Injury to the epidermis triggers a homeostatic response designed to rapidly seal the wound site and restore the skin. (Coulombe, 1997; Martin, *Science* 276:75-81, 1997; hereby incorporated by reference in its entirety). A blood clot rapidly forms whenever the injury event results in the destruction of capillary vessels in the upper dermis, providing a temporary physical barrier and a source of chemotactic signals that attract various types of inflammatory cells to the site. (Coulombe, 1997) This is followed by migration of resident skin cell types from the proximal intact tissue into the wound site, and their proliferation at the wound margins. Wound closure is achieved by the combined action of keratinocyte migration into the wound site and contraction of specialized fibroblasts in the tissue underneath the wound site, which pulls the edges of the wound closer together. Inefficient remodeling of the wound bed leaves a scar, and in severe cases can lead to loss of tissue function at the wound site. The ability to survive injury to the skin over a significant surface area is directly dependent upon the efficiency with which an epithelial lining conferring appropriate barrier function is restored over the wound site. (*Id.*)

Recruitment of resident cells from the healthy tissue surrounding the wound site, and the coordination of their migration, proliferation, and differentiation represent key features in the response of stratified epithelia to injury. (*Id.*) Considerable efforts have been dedicated to define the signals responsible for these phenomena. The resulting list of growth factors and cytokines shown to be present and active at the wounded skin site is lengthy (*Id.*) However, the mechanisms responsible for

completion of the repair response remain unknown. A method for accelerating the proliferation of epithelial stem cells ("ESC") and keratinocytes would promote a more rapid and efficient cellular response to stratified epithelial injury, and thus decrease scarring and loss of function at the injury site.

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Summary of the Invention

In one aspect, the present invention provides methods that promote ESC and keratinocyte proliferation by contacting the cells with angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII) analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation of ESC and keratinocytes, wherein the improvement comprises addition to the cell culture medium of an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists.

In a further aspect, the present invention provides kits for the propagation of ESC and keratinocytes, wherein the kits comprise an effective amount of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists, and instructions for using the amount effective of active agent as a cell culture medium supplement. In a preferred embodiment, the kit further comprises a cell culture media. In another preferred embodiment, the kit further comprises a sterile container for cell culturing.

Brief Description of the Figures

Figure 1 is a graph demonstrating the effect of AII fragments and analogues on keratinocyte proliferation.

Figure 2 is a graph demonstrating the effect of AII fragments, analogues, and fragment analogues on keratinocyte proliferation on Day 7 post-epithelial burn.

Detailed Description of the Preferred Embodiments

All cited patents, patent applications and references are hereby incorporated by reference in their entirety.

As defined herein, the term "epithelial stem cell (ESC)" refers to cells that are long-lived, relatively undifferentiated, have a great potential for cell division, and are ultimately responsible for the homeostasis of epithelium. Cells of this type include, but are not limited to, those described in U.S. Patent No. 5,556,783; U.S. Patent No. 5,423,778; Rochat et al., *Cell* 76:1063 (1994); Jones et al. *Cell* 73:713 (1993); Jones et al., *Cell* 80:83 (1995); all references hereby incorporated in their entirety. As defined herein, "proliferation" encompasses both cell self renewal and cellular proliferation with accompanying differentiation.

Unless otherwise indicated, the term "active agents" as used herein refers to the group of compounds comprising angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof and AII AT_2 type 2 receptor agonists.

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of

wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen (*Circulation Research* 60:786-790 (1987); Clouston et al., *Genomics* 2:240-248 (1988); Kageyama et al., *Biochemistry* 23:3603-3609; Ohkubo et al., *Proc. Natl. Acad. Sci.* 80:2196-2200 (1983); all references hereby incorporated in their entirety). The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [SEQ ID NO:37]. AII is a known pressor agent and is commercially available.

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) 1989; Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988); Naftilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem.* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer and Owens, *Circ. Res.* 70:820 (1992); Wolf, et al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141

(1985); LeNoble, et al., Eur. J. Pharmacol. 195:305-6 (1991). Additionally, AII and angiotensin III analogs and fragments thereof have been shown to be effective in tissue repair. (U.S. Patent No. 5,629,292; International Application No. WO 95/08565; International Application WO 95/08337; International Application No. WO 96/39164; all references hereby incorporated in their entirety.) AII has also been shown to increase cellular proliferation in hair follicles in the area of a thermal injury. (Rodgers et al., J. Burn Care Rehabil. 18:381-388 (1997).

The effect of AII on a given cell type has been hypothesized to be dependent, in part, upon the AII receptor subtypes the cell expresses (Shanugam et al., *Am. J. Physiol.* 268:F922-F930 (1995); Helin et al., *Annals of Medicine* 29:23-29 (1997); Bedecs et al., *Biochem J.* 325:449-454 (1997)). These studies have shown that AII receptor subtype expression is a dynamic process that changes during development, at least in some cell types (*Id.*). AII activity is typically modulated by either or both the AT1 and AT2 AII receptors. However, AII has recently been shown to stimulate proliferation of primary human keratinocytes via a non-AT1, non-AT2 receptor. (Steckelings et al., Biochem. Biophys. Res. Commun. 229:329-333 (1996)). These results underscore the cell-type (ie: based on receptor expression) specific nature of AII activity.

Other data suggests that the AII fragment AII(1-7) acts through a receptor(s) that is distinct from the AT1 and AT2 receptors which modulate AII activity. (Ferrario et al., J. Am. Soc. Nephrol. 9:1716-1722 (1998); Iyer et al., Hypertension 31:699-705 (1998); Freeman et al., Hypertension 28:104 (1996); Ambuhl et al., Brain Res. Bull. 35:289 (1994). Thus, AII(1-7) activity on a particular cell type cannot be predicted based solely on the effect of AII on the same cell type.

Based on all of these studies, it is not known whether angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof, and/or AII AT_2 type 2 receptor agonists accelerate the proliferation of epithelial stem cells or keratinocytes.

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) is p-aminophenylalanine6-AII ["(p-NH_2-Phe)6-AII)"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, BBRC 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992); Prescott, et al., *Am. J. Pathol.* 139:1291-1296 (1991); Kauffman, et al., *Life Sci.* 49:223-228 (1991); Viswanathan, et al., *Peptides* 13:783-786 (1992); Kimura, et al., *BBRC* 187:1083-1090 (1992).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et al., *Hypertension* 19(Supp. II):II-49-II-55 (1992); Edwards and Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673 (1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et a., *Br. J. Pharmacol.* 111:652-654 (1994).

As hereinafter defined, a preferred class of AT2 agonists for use in accordance with the present invention comprises AII, AII analogues or active fragments thereof having p-NH-Phe in a position corresponding to a position 6 of AII. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the requisite AT2 agonist activity are further contemplated for use in accordance with the present invention.

The active AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention comprise a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys,

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr, while Lys has also been found effective at this residue;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer, azaTyr, and Ala;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the sequence is not AII.

Particularly preferred embodiments of this class of compounds are SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:38.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R^6 is p-NH_2-Phe.

Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AIII or AII(2-8), Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro {SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-

Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

Another class of particularly preferred compounds in accordance with the present invention consists of those with the following general structure:

R1-Arg-R2-Tyr-R3-His-Pro-R4

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Ala, Ile, Leu, norLeu, and Val;

R4 is selected from the group consisting of Ile, Phe, and H; and

and wherein the active agent is not AII.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer, azaTyr, and Ala;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

R^2-R^3-Tyr-R^5-His-Pro-Phe [SEQ ID NO:16]

wherein R^2, R^3 and R^5 are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

Me^2Gly	N,N-dimethylglycyl
Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
azaTyr	aza-α'-homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid

Sar	N-methylglycyl (sarcosine)

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., *Pharmacological Reviews* 26:69 (1974). In general, it is believed that neutral side chains in position R^3, R^5 and R^7 may be involved in maintaining the appropriate distance between active groups in positions R^4, R^6 and R^8 primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions R^3, R^5 and R^8 may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R^2 may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R^2.

For purposes of the present invention, it is believed that R^3 may be involved in the formation of linear or nonlinear hydrogen bonds with R^5 (in the gamma turn model) or R^6 (in the beta turn model). R^3 would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R^3 may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr.

With respect to R^4, conformational analyses have suggested that the side chain in this position (as well as in R^3 and R^5) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R^4 is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. In this position, Tyr is

particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*). It has also been found that R^4 can be Ala.

In position R^5, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R^5, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R^6 is His, Arg or 6-NH_2-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor, aromatic character) are believed to contribute to its particular utility as R^6. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R^7. Similarly, it is presently considered that R^7 should be Pro in order to provide the most desirable orientation of R^8. In position R^8, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2

Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20

Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27
Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr$(PO_3)_2$-Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35

The polypeptides of the instant invention may be synthesized by any conventional method, including, but not limited to, those set forth in J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, *Hormonal Proteins and Peptides*, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the

crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

Preferably, peptides are synthesized according to standard solid-phase methodologies, such as may be performed on an Applied Biosystems Model 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics, either by solid phase methodologies or in liquid phase, are well known to those skilled in the art.

In one aspect of the present invention, a method of increasing *in vitro* and *ex vivo* ESC and keratinocyte proliferation by exposure to angiotensinogen, AI, AI analogues, and/or AI fragments and analogues thereof, AII analogues, AII fragments and analogues thereof, and/or AII AT_2 type 2 receptor agonists ("active agents"), is disclosed, either alone or in combination with other growth factors and cytokine. Examples of such growth factors and cytokines include, but are not limited to lymphokines, interleukins - 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, keratinocyte growth factor, tumor necrosis factor, epidermal growth factor ("EGF"), HB-EGF, fibroblast growth factors 1, 2 and 4, and transforming growth factor alpha. Experimental conditions for the isolation, purification, *ex vivo* growth and *in vivo* mobilization of ESC and keratinocytes have been reported (U.S. Patent No. 5,423,778; U.S. Patent No. 5,561,107; U.S. Patent No. 5,686,307; Rheinwald et al., in: The Formation of Keratinizing Colonies from Cells. Cell G. 331-343, 1975; all references hereby incorporated in their entirety).

In a preferred embodiment of the invention, ESC and keratinocytes are obtained by trypsinization of skin samples from patients undergoing plastic surgery, as

previously described (Svendsen et al., *Pharmacology and Toxicology* 80:49-56 (1997)). In another preferred embodiment, ESC are isolated from the upper hair follicle, as described in U.S. Patent No. 5,556,783, herein incorporated by reference in its entirety.

Isolated ESC and keratinocytes are then cultured under appropriate growth conditions, in the presence of the active agents of the invention. Cell proliferation is assessed at various time points during culture using methods well known in the art, including, but not limited to, measuring the rate of DNA synthesis according to the method of Nakamura and coworkers (Nakamura et al., *J. Biochem.* (Tokyo) 94:1029 (1982); Nakamura et al., Biochem. Biophys. Res. Comm. 122:1450 (1984)) Trypan blue dye exclusion/hemocytometer counting (Omori et al., *Hepatology* 26:720 (1997)), or flow cytometry (Drakes, 1997).

In a preferred embodiment, ESC and keratinocytes are suspended in standard culture medium, including but not limited to, keratinocyte-basal growth medium (Clonetics Corp., San Diego, CA) and incubated in the presence of, preferably, between about 0.1 ng/ml and about 10 mg/ml of the active agents The cells are expanded for a period of between 8 and 21 days, and cellular proliferation is monitored by measuring the rate of DNA synthesis (Nakamura et al., 1982; Nakamura et al., 1984). ESC and keratinocyte proliferation is measured by ^{3}[H]thymidine incorporation as described in Yang et al., *J. of Investig. Dermatol.* 107:367-372 (1996). Alternatively, ESC and keratinocyte cell proliferation is assessed periodically by immunohistochemistry using an antibody directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, such as proliferating cell nuclear antigen (PCNA or cyclin; Zymed Laboratories) (Rodgers et al., 1997).

Differentiation of keratinocytes to squames is detected by measuring the expression of typical differentiated keratinocyte markers including, but not limited to, transglutaminase type I (TG-I) (Svendsen et al., 1997) and keratin (Martin et al., 1997) Methods of detecting these markers include, but are not limited to, Northern blot analyses or reverse transcriptase-polymerase chain reaction (RT-PCR) with marker-specific DNA primers (Song et al., *Biochem. Biophys. Res. Commun.* 235:10-14 (1997); Takahashi et al., *J. Biol. Chem.* 270:18581-92 (1995)), and antibody detection (The Binding Site, San Diego, CA).

In a further preferred embodiment, ESC and keratinocytes that have been cultured in the presence of active agents, are used for *ex vivo* ESC- and keratinocyte-directed gene therapy, as described in Morgan et al., Science 237:1476-1479 (1987), and Tenmer et al., *FASEB J.* 4:3245-3250 (1990). Prior to *ex vivo* ESC- or keratinocyte-directed gene therapy, the cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7×10^6 and 50×10^6 cells per ml in an appropriate medium and re-introduced into a subject, as described below.

In another aspect of the present invention the active agents are used to increase *in vivo* ESC and keratinocyte proliferation, which can be measured by taking biopsy samples and using the methods described above. In a preferred embodiment, biopsy samples are taken between about 1 day to about 2 weeks after treatment.

For use in increasing the proliferation of ESC and keratinocytes, the active agents may be administered by any suitable route, including orally, parentally, by inhalation spray, rectally, transdermally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles.

The term parenteral as used herein includes, subcutaneous, intravenous, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally.

The active agents may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions), and may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While the active agents can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other compounds. When administered as a combination, the active agents and other compounds can be formulated as separate compositions that are given at the same time or different times, or the active agents and other compounds can be given as a single composition.

For administration, the active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as

glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

The dosage regimen for increasing *in vivo* proliferation of ESC and keratinocytes with the active agents is based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between 0.1 ng/kg and 10 mg/kg of the active agents per body weight are useful for all methods of use disclosed herein.

In a preferred embodiment of the present invention, the active agents are administered transdermally or topically. A suitable transdermal or topical dose of active ingredient of the active agents is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily. For transdermal administration, the active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

A formulation suitable for topical administration includes liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

Transdermal means including, but not limited to, transdermal patches may be utilized to deliver the active agents to the treatment site. Transdermal formulations may be prepared by incorporating the active agent in a thixotropic or gelatinous carrier including, but not limited to, a cellulose medium, e.g., methyl cellulose or hydroxyethyl

cellulose, with the resulting formulation then being packed in a transdermal device adapted to be secured in dermal contact with the skin of a wearer.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation of ESC and keratinocytes, wherein the improvement comprises addition to the cell culture medium of an effective amount of the active agents as described above. Any cell culture media that can support the growth of ESC and keratinocytes can be used with the present invention. Such cell culture media include, but are not limited to Keratinocyte Basal Growth Media, Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, and RPMI Medium, or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media and serum supplements are commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO)

In a further aspect, the present invention provides kits for the propagation of ESC and keratinocytes, wherein the kits comprise an effective amount of the active agents of the invention, and instructions for using the amount effective of active agent as a cell culture medium supplement.

In a preferred embodiment, the kit further comprises cell culture growth media. Any cell culture media that can support the growth of ESC and keratinocytes can be used with the present invention. Examples of such cell culture media are described above.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement.

In a further preferred embodiment, the kit further comprises a sterile container. The sterile container can comprise either a sealed container, such as a cell culture flask, a roller bottle, or a centrifuge tube, or a non-sealed container, such as a cell culture plate or microtiter plate (Nunc; Naperville, IL).

In a preferred embodiment, the kit further comprises an antibiotic supplement for inclusion in the reconstituted cell growth medium. Examples of appropriate antibiotic supplements include, but are not limited to actimonycin D, Fungizone®, kanamycin, neomycin, nystatin, penicillin, streptomycin, or combinations thereof (GIBCO).

The present invention, by providing methods for enhanced proliferation of ESC and keratinocytes, will be clinically useful in accelerating ESC and keratinocyte growth during wound healing and skin transplantation, using either natural skin or a living skin equivalent. The method of the present invention also increases the potential utility of ESC and keratinocytes as vehicles for gene therapy, by more efficiently providing a large number of such cells for transfection, and also by providing a more efficient means to rapidly expand transfected ESC and keratinocytes.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention.

Example 1. Effect of AII and AII analogues and analogue fragments on keratinocyte proliferation in vitro as measured by cell number

Normal human keratinocytes were purchased from Clonetics (San Diego, CA) and cultured according to the manufacturer's instructions in Keratinocyte Growth Medium (KGM; Keratinocyte Basal Medium containing human recombinant epidermal growth factor, human recombinant insulin, hydrocortisone, epinephrine, prostaglandin E2 and antibiotics) until confluent. Upon reaching confluence, the cells were trypsinized from the culture flask and re-seeded into 24 well plates at 100 cells per well in KGM. After allowing adherence of the cells for 24 hours, the cells were placed in KBM and 10 μg/ml of either AII (SEQ ID NO:1), AII(1-7) (SEQ ID NO:4), or Ala4AIII (Arg-Val-Tyr-Ala-His-Pro-Phe) (SEQ ID NO:18) was added to the wells. As shown in Table 1, exposure of keratinocytes to the various peptides increased the number of keratinocytes per well over KBM alone.

TABLE 1. Effects of AII(1-7) and Ala3-AIII on In Vitro Keratinocyte Proliferation

Peptide Tested	Cells/Well (Mean +/- SEM)
Control	70 +/- 25.2
AII (SEQ ID NO:1)	767 +/- 145.3
AII(1-7) (SEQ ID NO:4)	1267 +/- 371.2
Ala3-AIII (SEQ ID NO:39)	1333 +/- 333.0

Example 2. Effect of AII and AII analogues and analogue fragments on keratinocyte proliferation in vitro as measured by thymidine incorporation

Normal human keratinocytes were purchased from Clonetics (San Diego, CA) and cultured in Keratinocyte Growth Medium (KGM) as described in Example 1, until confluence at 37°C in 5% CO_2. The cells were harvested at confluence with 0.02% trypsin/0.05% ETA. Thereafter, the cells were seeded into 24 well plates at a density of 50,000 cells per well. After 12 hours, the KGM was replaced with basal medium (KBM) and the cells were cultured to quiescence for 24 hours. Cells were then cultured with 10 μg/ml of various peptides (AII, AII(1-7), or Ala4-AIII) for 24 hours, after which 1 μCi [^{3}H] thymidine was added to each well. The cultures were then continued at 37°C in 5% CO_2 for an additional 12 hours. The cells were then washed 3 times with PBS (pH 7.2) to remove unincorporated radioactivity. The cells were then washed once with 5% TCA, once with 100% ethanol, and then lysed in 1M NaOH, and 200 μl aliquots of the lysate were counted in a scintillation counter. The data are presented in Figure 1 and demonstrate that all peptides tested increased thymidine incorporation in comparison with medium control.

Example 3. Effect of AII analogues and fragments and analogue fragments on in vivo keratinocyte proliferation

Male Hartley guinea pigs weighing approximately 500 grams were purchased from Charles Rivers Laboratory (Charles Rivers, MA) and anesthetized by intramuscular injection of 14 mg/kg Rompun and 130 mg/kg Ketamine. Hair was then removed from the dorsal surface through shaving with animal clippers followed by treatment with thioglycollate depilatory. After hair removal the area was twice scrubbed with betadine followed by a 70% ethanol scrub. Two burns were produced on

each guinea pig with an 18 mm solid brass rod, which was warmed in a 75°C water bath. One end of the brass rod was placed on the back of the guinea pig for 50 seconds. This procedure was repeated with different brass rods for each animal and each burn.

Each burn was treated with 10% low viscosity carboxymethylcellulose (CMC sodium salt, Sigma Chemical CO., St. Louis, MO) in 0.05 M phosphate buffer, pH 7.2, and sterilized by autoclaving, with and without either 1 mg/ml AII(1-7) (SEQ ID NO:4), Ala4-AIII (SEQ ID NO:18), Pro3-AII (SEQ ID NO:31), or Ile8-AII (Asp-Arg-Val-Tyr-Ile-His-Pro-Ile) (SEQ ID NO:38). Each burn was subsequently dressed with a Hilltop Chamber (Hilltop Research, Madison, WI) and covered with Tegaderm (Western Medical Supply, Arcadia, CA). The bandages were checked and changed daily for the first 5 days and every other day until necropsy on day 7 after injury. The guinea pigs were given 20 μg/kg bupronex (buprenophine hydrochloride) intramuscularly for pain on the day of injury and the first 3 days after injury.

On day 7 after thermal injury, the guinea pigs were euthanized, the burned areas were excised en bloc and the tissues were placed in 10% buffered formaldehyde solution overnight. The tissues were embedded in paraffin and 5 μm sections were prepared. The sections were processed for immunohistochemical analysis with a primary antibody to cyclin (MIB-1) followed by recognition of the primary antibody with a DAKO kit (see following text).

The paraffin embedded sections were baked in an oven overnight at 60°C. De-paraffinization was performed by four 5 minute incubations in "fresh" xylene followed by two 5 minute incubations with 100% ethanol, two 5 minute incubations with 95% ethanol, and one 5 minute incubation with water. Endogenous peroxidase activity was then quenched with 0.3% H_2O_2 in water. Antigen retrieval was performed following

the protocol of Rodgers et al. (1997) with minor modifications. In brief, the slides were placed in plastic coplin jars in 5% urea in 0.1 mol/L Tris buffer (pH 9.0). The slides were then placed in a microwave oven and set at maximal power for 3 minutes. The antigen retrieval solution was replenished with distilled water in cases when evaporation was excessive and microwaved again for 5 minutes at 50% power. The slides were then allowed to cool for 10 minutes and were placed in phosphate-buffered saline solution (PBS) for 5 minutes.

Immunohistochemical staining was performed with the avidin-biotin-peroxidase conjugate method with some modifications. The slides were placed in a PBS bath for 5 minutes and then laid in humidified incubation chambers. Blocking for nonspecific antibody binding was performed by incubating the sections in 5% horse serum in PBS. The solution was decanted and replaced with the solution of primary antibody MIB-1, used at a dilution of 1:100 in PBS, and incubated for 60 minutes at room temperature. The slides were washed with PBS for 5 minutes and then incubated with a biotinylated horse-anti-mouse antibody. After a 5 minute wash in PBS, the avidin-biotin complex was applied to the slides at a dilution of 1:100 in PBS at room temperature and was incubated for 1 hour.

After a final PBS rinse, the sections were incubated in 0.06% 3,3'-diaminobenzidine in PBS with 0.03% hydrogen peroxide for 5 minutes. After a counterstain was performed in modified Harris' hematoxylin-eosin the sections were dehydrated and coverslipped with Permount.

With an Olympus Vanox-S AH-2 dissecting microscope and a magnification power of 100X, each section of the biopsy specimen was separated into either (a) areas on the burn edge or (b) the actual burn areas. The area of the burn was excised and

serially cut into three to five sections of 4-5 mm in thickness. The entire area of the burn and edge of the burn was embedded and examined histologically. The cells that stained with the MIB-1 antibody were a distinct brown color. To count the MIB-1 stained cells, each section on the slide was separated into individual 100X fields. Each field was then determined to be either a section on the edge of the burn or a part of the burn area itself. An edge was indicated by a positive stain showing brown epithelial cells along the edge of the section. The brown cells in the basal layer of the epidermis (proliferating keratinocytes) located at the 100X field adjacent to the burn site were counted one at a time.

The depth of the burns was determined to be deep partial-thickness to full-thickness by (1) analysis of vessel patency with intraaortic injection of India Ink, and (2) the appearance of the cells in the hair follicles by microscopic analysis of hematoxylin-eosin-stained sections. This analysis revealed that most of the pre-existing blood vessels and cells were destroyed in the burn site, although the injuries did not extend through the panniculus carnosus.

The results (Figure 2) demonstrate that daily administration of AII(1-7) (SEQ ID NO:4), Ala3-AIII (SEQ ID NO:39), Pro3-AII (SEQ ID NO:31), and Ile8-AII (SEQ ID NO:38) all increased the number of cyclin-positive cells *in vivo* in comparison with placebo controls on individual animals.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for promoting epithelial stem cell or keratinocyte proliferation comprising contacting epithelial stem cells or keratinocytes with an amount effective to promote proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the active agent is not AII.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39.

3. The method of claim 1 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:38, and SEQ ID NO:39.

4. The method of claim 1 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

5. An improved cell culture medium for promotion of epithelial stem cell or keratinocyte cell proliferation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation of epithelial stem cells or keratinocytes of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

and wherein the active agent is not AII.

6. The improved cell culture medium of claim 5 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39.

7. The improved cell culture medium of claim 5 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:38, and SEQ ID NO:39.

8. The improved cell culture medium of claim 5 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

9. A kit for promoting epithelial stem cell and keratinocyte proliferation comprising:

(a) an amount effective to promote epithelial stem cell or keratinocyte proliferation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group,

R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc,

R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc, Lys and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr(PO_3)$_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group,

wherein the active agent is not AII; and

(b) instructions for using the amount effective of active agent to promote epithelial stem cell or keratinocyte proliferation.

10. The kit of claim 9 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36; SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39.

11. The kit of claim 9 wherein the active agent is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:38, and SEQ ID NO:39.

12. The kit of claim 9 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

13. A method for promoting epithelial stem cell or keratinocyte proliferation comprising contacting epithelial stem cells or keratinocytes with an amount effective to promote proliferation of at least one active agent comprising a sequence consisting of an active agent comprising a sequence of the following general formula:

R1-Arg-R2-Tyr-R3-His-Pro-R4

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Ala, Ile, Leu, norLeu, and Val;

R4 is selected from the group consisting of Ile, Phe, and H; and

wherein the active agent is not AII.

14. The method of claim 13 wherein the active agent is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:38.

15. The method of claim 13 wherein the concentration of active agent is between about 0.1 ng/kg and about 10.0 mg/kg.

16. An improved cell culture medium for promotion of epithelial stem cell or keratinocyte cell proliferation, wherein the improvement comprises addition to the cell culture medium an amount effective to increase proliferation of epithelial stem cells or keratinocytes of at least one active agent comprising a sequence consisting of an active agent comprising a sequence of the following general formula:

R1-Arg-R2-Tyr-R3-His-Pro-R4

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Ala, Ile, Leu, norLeu, and Val;

R4 is selected from the group consisting of Ile, Phe, and H; and

wherein the active agent is not AII.

17. The improved cell culture medium of claim 16 wherein the active agent is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13,

SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:38.

18. The improved cell culture medium of claim 16 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

19. A kit for promoting epithelial stem cell and keratinocyte proliferation comprising:

(a) an amount effective to promote epithelial stem cell or keratinocyte proliferation of at least one active agent comprising a sequence consisting of an active agent comprising a sequence of the following general formula:

R1-Arg-R2-Tyr-R3-His-Pro-R4

wherein R1 is selected from the group consisting of H and Asp;

R2 is selected from the group consisting of Val and Pro;

R3 is selected from the group consisting of Ala, Ile, Leu, norLeu, and Val;

R4 is selected from the group consisting of Ile, Phe, and H; and

wherein the active agent is not AII; and

(b) instructions for using the amount effective of active agent to promote epithelial stem cell or keratinocyte proliferation.

20. The kit of claim 19 wherein the active agent is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:26, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:38.

21. The kit of claim 19 wherein the concentration of active agent is between about 0.1 ng/ml and about 10.0 mg/ml.

FIG. 1

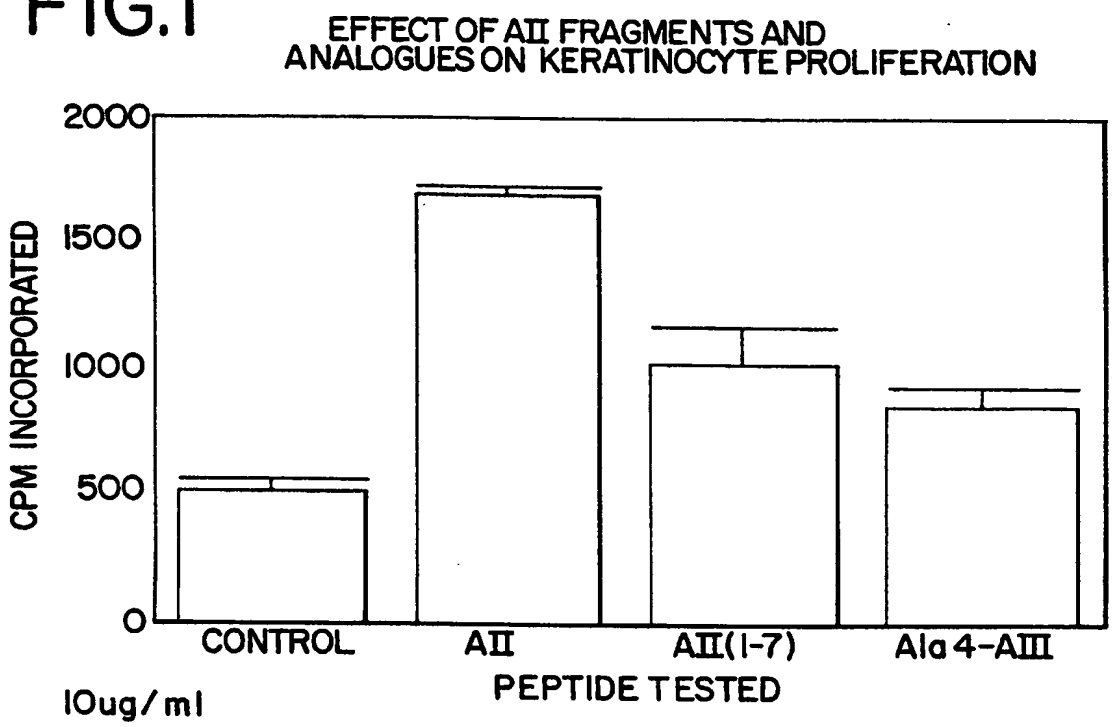
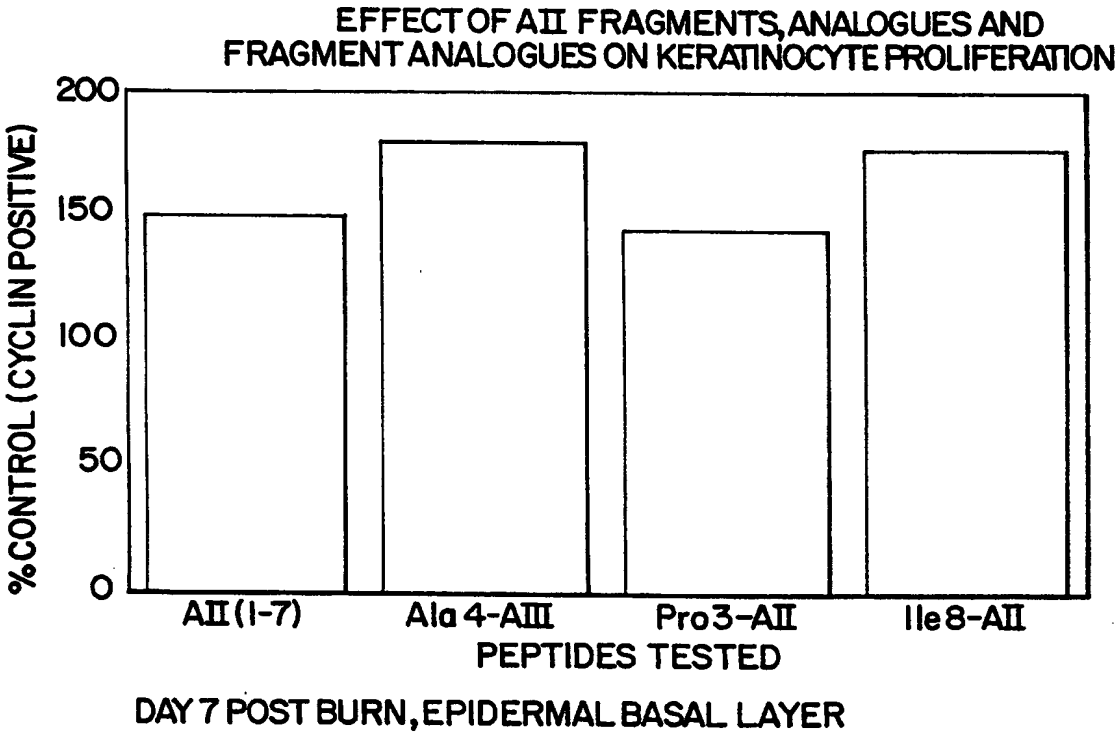

EFFECT OF AII FRAGMENTS AND
ANALOGUES ON KERATINOCYTE PROLIFERATION
CPM INCORPORATED
2000
1500
1000
500
0
CONTROL
AII
AII(1-7)
Ala 4-AIII
PEPTIDE TESTED
10ug/ml

FIG.2


EFFECT OF AII FRAGMENTS, ANALOGUES AND
FRAGMENT ANALOGUES ON KERATINOCYTE PROLIFERATION
200
150
100
50
0
% CONTROL (CYCLIN POSITIVE)
AII (1-7)
Ala 4-AIII
Pro3-AII
Ile8-AII
PEPTIDES TESTED
DAY 7 POST BURN, EPIDERMAL BASAL LAYER

SEQUENCE LISTING

<110> Kathleen Rodgers and Gere diZerega

<120> Method for Promoting Hematopoietic and Mesenchymal Cell Proliferation and Differentiation

<130> 98,028-B

<140> To be assigned

<141> To be assigned

<160> 38

<170> PatentIn Ver. 2.0

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<212> PRT

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1 5

<210> 3

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Val Tyr Ile His Pro Phe

1 5

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Arg Val Tyr Ile His Pro

1 5

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Val Tyr Ile His Pro

1 5

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Ile His Pro Phe

1

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1 5

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Asp Arg Val

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<223> Description of Artificial Sequence:AII analogue

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1

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Tyr Ile His Pro Phe

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Ser(Acetylated), MeGly, D-Arg, or D-Lys; Xaa at position 2
can be Val, Ala, Leu, Nle, Ile, Gly, Pro, Aib, Acp, or Tyr;
Xaa at position 4 can be Ile, Ala, Leu, Nle, Val, or Gly

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1 5

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1 5

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Asp Arg Val Tyr Ile Arg Pro Phe

1 5

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Asp Arg Val Tyr Ile His Ala Phe

1 5

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<211> 8

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Asp Arg Val Tyr Ile His Pro Tyr

1 5

<210> 30

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<223> Description of Artificial Sequence:AII Analogue 12

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Pro Arg Val Tyr Ile His Pro Phe

1 5

<210> 31

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Asp Arg Pro Tyr Ile His Pro Phe

1 5

<210> 32

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Asp Arg Val Tyr Ile His Pro Phe

1 5

<210> 33

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<223> Description of Artificial Sequence:AII Analogue 15

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1 5

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Asp Arg Val Tyr Xaa His Pro Phe

1 5

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Asp Arg Val Ser Tyr Ile His Pro Phe

1 5

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Asp Arg Val Tyr Ile Xaa Pro Phe

1 5

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Asp Arg Val Tyr Ile His Pro Phe His Leu

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Asp Arg Val Tyr Ile His Pro Ile

1 5